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Purification and properties of the glutamate oxaloacetate transaminase of the ectomycorrhizal fungus *Cenococcum geophilum*

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INTRODUCTION

The aminotransferases or transaminases are enzymes catalysing the transfer of an amino group from an amino acid (donor) to a keto acid (acceptor). The result is the formation of a new amino acid plus the keto acid analogue of the amino acid which originally served as the amino donor. Most transaminations are freely reversible processes.

The enzymatic transfer of amino groups plays an important part in many metabolic processes when the interconversion of nitrogen-containing molecules is involved. As shown in figure 1, nitrogen, following its initial assimilation into glutamate can be distributed to many other compounds, especially other amino acids by the action of aminotransferases (MIFLIN and LEA, 1980).

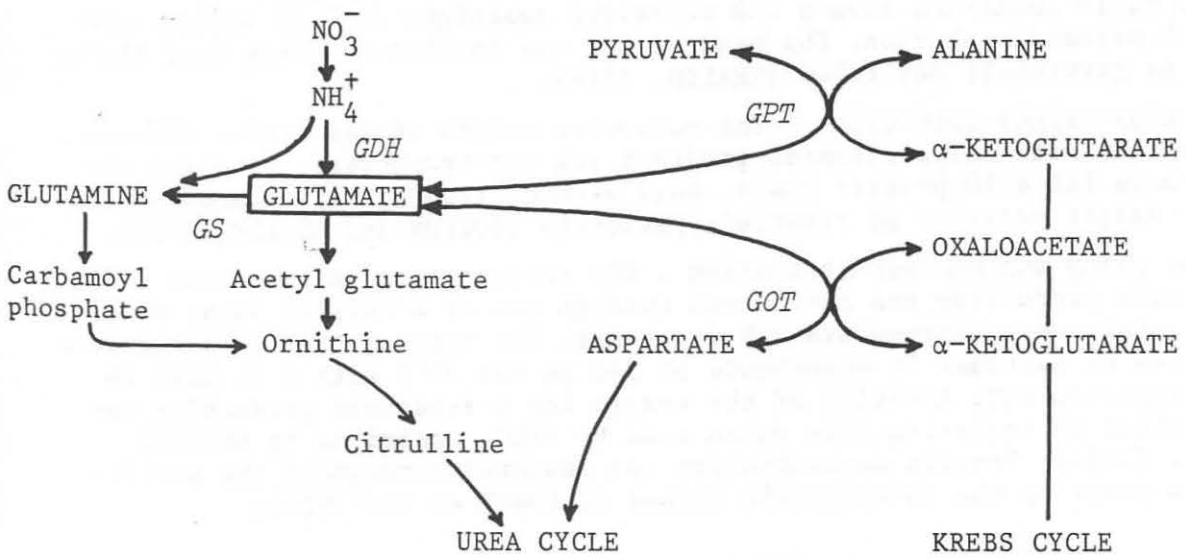

Figure 1: Scheme of a few major events in nitrogen metabolism in fungi.
GDH: glutamate dehydrogenase, GS: glutamine synthetase, GOT: glutamate oxaloacetate transaminase, GPT: glutamate pyruvate transaminase.

Glutamate is often the amino-donor substrate in transamination reactions. This compound can be converted to glutamine by glutamine synthetase (GS) and can also be the substrate of transaminases, glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT), to give rise to aspartate and alanine respectively. A competition between these two metabolic routes was demonstrated in *Neurospora crassa* (KANAMORI et al. 1982) and in *Cenococcum geophilum* (GENETET 1984).

This work is devoted to the study of the glutamate oxaloacetate transaminase (E.C. 2.6.1.1) which catalyzes the following reaction :
L-Aspartate + α-ketoglutarate $\rightleftharpoons$ Oxaloacetate + L-Glutamate

MATERIALS AND METHODS

Organism and purification techniques : *Cenococcum geophilum* was cultivated in Erlenmeyer flasks on Pachlewski's liquid medium at 25°C for 12 days.

Colonies were ground in a mortar with acid-washed sand in presence of 0.1 M K-phosphate buffer, pH 8 that contained Polyvinylpyrrolidone (10% of fresh weight of material). The crude homogenate was filtered through a bolting cloth and centrifuged at 40 000 g for 20 min. The supernatant was treated with solid ammonium sulfate and proteins precipitating between 70 and 90% of saturation were dissolved in 0.01 M K-phosphate buffer, pH 8. This extract was applied to a column packed with hydroxyapatite. Proteins were eluted with a linear gradient of the same buffer from 0.01 M to 0.5 M. Fractions with the highest activities were pooled and passed through a DEAE-cellulose column equilibrated with 0.01 M Tris-HCl buffer at pH 8. Proteins were eluted with a linear gradient from 0 to 0.5 M NaCl in Tris-HCl buffer. The active fractions were concentrated by diafiltration on Amicon PM 10 and filtrated on ACA 22.

The homogeneity of GOT was analysed by electrophoresis in small tubes (0.7 x 7 cm) containing 1.5 ml of 7% polyacrylamide gel. Runs were performed at 4 mA per tube. After electrophoresis the gels were stained for 2 h. in Coomassie Blue R and destained overnight in 7.5% acetic acid and 5% methanol solution. The band of GOT was located by using Fast Violet Blue as previously described (KHALID, 1984).

Molecular weight estimation : The molecular weight of the native GOT was determined with polyacrylamide gradient gel electrophoresis by using the Pharmacia PAA 4/30 precast slabs. Gels were calibrated with standard molecular weight proteins as reported previously (BOTTON and MSATEF, 1983).

Enzyme assay and protein estimation : GOT activity for oxaloacetate and glutamate production was determined through use of a coupled assay reaction catalyzed by NAD-malate dehydrogenase. The rate of NADH oxidation was measured by decrease in absorbance at 340 nm and 30°C with a Beckman DB spectrophotometer. Activity of the enzyme for L-aspartate production was determined by analysing this amino acid by HPLC, according to GENETET et al. (1984). Protein concentration was measured throughout the purification steps by the colorimetric method of LOWRY et al.(1951).

RESULTS AND DISCUSSION

Purification : Table 1 shows the details of the best preparation obtained to purify GOT from *C. geophilum* near homogeneity. The highest purification achieved was 175-fold with 20% recovery of initial total activity.

Table 1: *Purification of Glutamate oxaloacetate transaminase from Cenococcum geophilum.*

Purification step	Total Activity (nkat)	Specific Activity (nkat mg^{-1} protein)	Purification (Fold)	Yield (%)
Crude extract	5175	6.2	1	100
Ammonium sulfate (70-90% saturation)	4507	49	7.8	87
Hydroxyapatite	2873	57.4	9.2	55.5
DEAE-cellulose	2086	515.1	82.6	40.3
Gel filtration	1043	1087.3	175.5	20.1

Electrophoresis on 7% polyacrylamide gel revealed only a single form of GOT caracterized by a Rf of 0.22. Similar analytical results were obtained when the purified enzyme was subjected to electrophoresis on continuous gradient gels (data not shown).

The molecular weight of the native GOT was estimated to be near 140 000 dalton by electrophoresis on a calibrated polyacrylamide gradient gel (Fig.2).

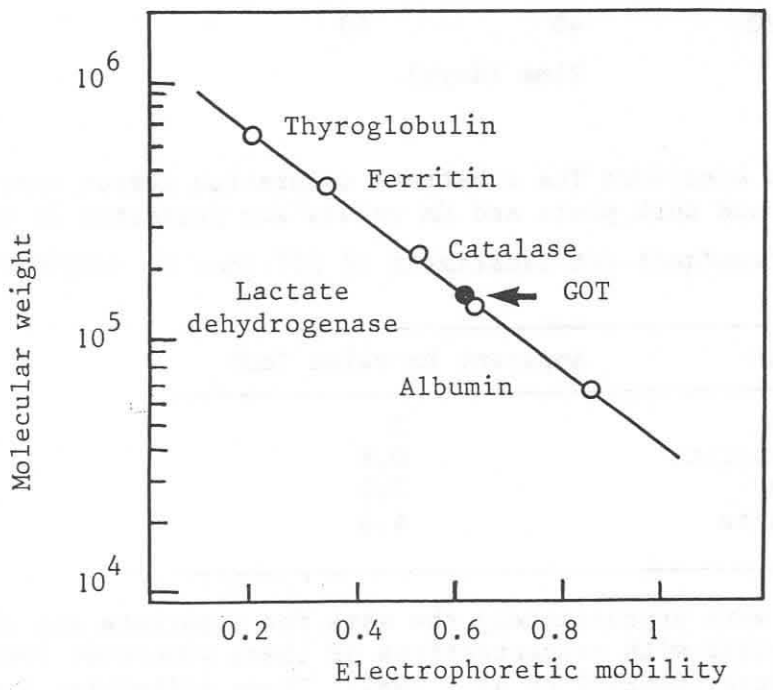

Figure 2: *Semilogarithmic plot of molecular weights of standard marker proteins and the purified GOT from C. geophilum as a function of Rf derived from polyacrylamide gradient gel electrophoresis.*

Catalytic properties:

Optimum pH: With phosphate and Tris-HCl buffers, maximum activity was at pH 7.5 for the formation of glutamate and at pH 6.2 for the formation of aspartate. These values are slightly different from those reported with other Ascomycetes including *Neurospora crassa* (MUNKRES, 1965) and *Sphaerostilbe repens* (BOUKROUTE, 1984).

Thermal stability: The purified enzyme remained fully active at 4°C for at least 15 days. The activity decreased very rapidly at -25°C. In both cases the presence of α-ketoglutarate 0.4 M preserved its activity much longer (Fig.3).

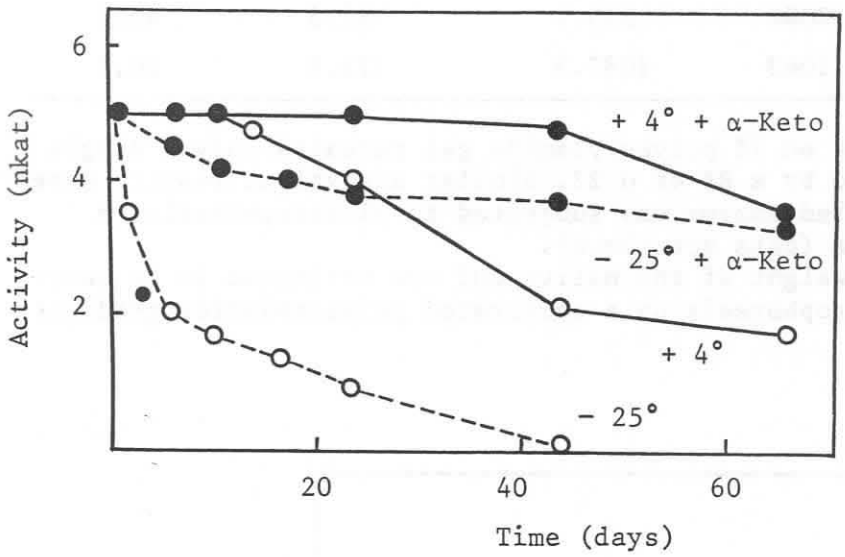

Figure 3: Thermal inactivation of GOT from C. geophilum

Substrate saturation kinetics: The substrate saturation curves were analysed by Lineweaver and Burk plots and Km values are presented in table 2.

Table 2: Michaelis constants for substrates of GOT from C. geophilum

Substrate	Apparent Km value (mM)
Aspartate	2
α-ketoglutarate	0.8
Glutamate	3.4
Oxaloacetate	4.8

Michaelis constants were approximately the same for aspartate and glutamate, and are consistent with concentrations of these compounds found in the cells of the fungus (GENETET et al., 1984). These affinities for the substrates show no clearly preferred reaction direction and are compatible with recent ^{15}N and ^{13}C nuclear magnetic resonance data obtained from *C. geophilum* (MARTIN, 1985; MARTIN et al., 1985).

Pyridoxal Phosphate requirement: The enzyme showed an appreciable increase in activity when pyridoxal phosphate was added to the reaction mixture (Fig. 4). This result indicates that coenzyme moiety was loosely bound to the apoenzyme of this aminotransferase. The linkage of this cofactor to fungus GOT is sometimes much tighter (BOUKROUTE, 1984).

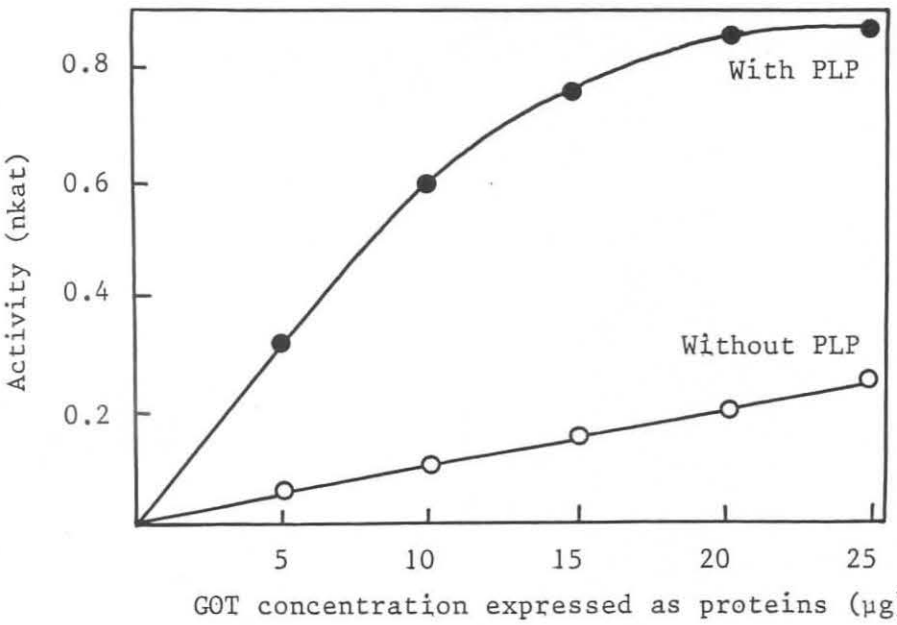

Figure 4: Effect of Pyridoxal Phosphate (PLP) on the activity of different concentrations of GOT of C. geophilum

In conclusion, the purified GOT from the ectomycorrhizal fungus *C. geophilum* is similar, with respect to the physicochemical and kinetics properties, to the enzyme of other plants (GIVAN, 1980). However slight differences exist between glutamate oxaloacetate transaminases of different fungi. Immunological techniques are now in progress to investigate the location of this enzyme in the cells and to quantify the protein in order to investigate its importance in controlling the nitrogen nutrition of the mycorrhizae.

BIBLIOGRAPHY

BOTTON B., MSATEF Y., 1983. *Physiol. Plant.*, 59, 438-444.

BOUKROUTE A., 1984. *DEA, Université de Nancy I*, 48p.

GENETET I., MARTIN F., STEWART G.R., 1984. *Plant Physiol.*, 76, 395-399.

GIVAN C.V., 1980. In: *The Biochemistry of Plants. A comprehensive treatise* Vol. 5, 329-357, (ed. B.J. MIFLIN), Academic Press New York.

KANAMORI K., LEGERTON T.L., WEISS R.L., ROBERTS J.D., 1982. *J. Biol. Chem.*, 257, 14168-14172.

KHALID A., 1984. *DEA. Université de Nancy I*, 54p.

LOWRY O.H., ROSEBROUGH N.H., FARR A.L., RANDALL R.J., 1951. *J. Biol. Chem.* 193, 265-275.

MARTIN F., 1985. *FEBS Lett.*, 182, 350-354.

MARTIN F., CANET D., MARCHAL J.P., 1985. *Plant Physiol.*, 77, 499-502.

MIFLIN B.J., LEA P.J., 1980. *The Biochemistry of Plants. A comprehensive treatise*. Vol. 5, 169-202, (ed. B.J. MIFLIN), Academic Press New York

MUNKRES K.D., 1965. *Arch. Biochem. Biophys.*, 112, 340-346.